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HPRT_{Sardinia}: A new point mutation causing HPRT deficiency without Lesch–Nyhan disease

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Abstract

Hypoxanthine-guanine phosphoribosyltransferase (HPRT) deficiency always causing hyperuricemia presents various degrees of neurological manifestations, the most severe which is Lesch–Nyhan syndrome. The HPRT gene is situated in the region Xq26–q27.2 and consists of 9 exons. At least 300 different mutations at different sites in the HPRT coding region from exon 1 to exon 9 have been identified. A new mutation in the HPRT gene has been determined in one patient with complete deficiency of erythrocyte activity, with hyperuricemia and gout but without Lesch–Nyhan disease. Analysis of cultured fibroblasts revealed minimal residual HPRT activity mainly when guanine was the substrate. Genomic DNA sequencing demonstrated patient's mother heterozygosity for the mutation and no mutation in her brother. The mutation consists in a C→T transversion at cDNA base 463 (C463T) in exon 6, resulting in proline to serine substitution at codon 155 (P155S). This mutation had not been reported previously and has been designated HPRT_{Sardinia}. The mutation identified in this patient allows some expression of functional enzyme in nucleated cells such as fibroblasts, indicating that such cell type may add further information to conventional blood analysis. A multicentre survey gathering patients with variant neurological forms could contribute to understand the pathophysiology of the neurobehavioral symptoms of HPRT deficiency.

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1. Introduction

Lesch–Nyhan disease (LND) is a complex syndrome presenting with uric acid urinary stones, spastic cerebral palsy, choreoatetosis and neurological dysfunction including

self-destructive biting of fingers [1]. It is an inborn X-linked disease caused by complete deficiency of the enzyme hypoxanthine-guanine phosphoribosyltransferase due to mutation in the encoding gene located in X-chromosome. Only males are affected and females usually behave as heterozygous carriers, though a very limited number of affected females bearing double mutations have been described [2]. Different estimated incidences have been reported for LND, 1:200.000 [3], or 1:380.000 to 1:100.000 male births [4]. The deficiency is ubiquitous and affects all races. It has been estimated that one third of LND males would represent new mutations.

The genetically determined deficiency of this enzyme is associated with variant clinical phenotypes mainly depending on the degree of deficiency, in which neurological

Abbreviations: HPRT, hypoxanthine-guanine phosphoribosyltransferase; LND, Lesch–Nyhan disease; APRT, adenine phosphoribosyltransferase; Ade, adenine; Hyp, hypoxanthine; Gua, guanine; UAC, Uric acid; XAN, xanthine

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abnormalities may be absent (previously called Kelley Seegmiller Syndrome) [5], mild (some degree of mental retardation, spasticity, dystonia) or very severe such as in LND, usually associated with virtually complete HPRT deficiency.

The HPRT locus is a constitutively expressed house-keeping gene characterized by a notably higher level of expression in mammalian brain and testes. The HPRT-encoding gene is situated in the long arm of the X chromosome in the region q26–q27.2 and consists of 9 exons and 8 introns totalling 57 kb. This gene is transcribed to produce an mRNA of 1.6 kb, which contains a protein-encoding region of 654 nucleotides [6,7], 657 comprising termination codon (UAA). The enzyme is 217 amino acid long with 24,470 molecular weight and undergoes two post translational modifications: acetylation of the NH₂-terminal alanine and deamination of asparagine 106 [8]. The native enzyme is normally a tetramer of identical subunits [7].

Most patients with HPRT deficiency have nearly normal levels of HPRT-specific mRNA, thus the locus is particularly amenable to analysis using polymerase chain reaction amplification of reverse transcribed RNA [2,9]. Sequencing the HPRT gene in genomic DNA provides direct diagnostic analysis not dependent on the expression of the mature HPRT mRNA.

The marked genetic heterogeneity of HPRT deficiency is well known and identification of mutations has been performed at the RNA and DNA level. Mutations include single DNA base substitutions, frameshift mutations, large and small DNA deletions, DNA base insertions and errors in mRNA splicing. At least 300 different mutations at different sites in the HPRT coding region from exon 1 to exon 9 have been identified [2,10].

This paper presents a biochemical and molecular study conducted on a patient with virtually complete deficiency of erythrocyte HPRT but not presenting the neurological symptoms typical of LND. At molecular level, a mutation was found in exon 6. Biochemical studies in patient's fibroblasts identified residual phosphoribosyltransferase activity mainly acting on guanine.

2. Materials and methods

2.1. Materials

Reagents of analytical grade were purchased from SIGMA (St. Louis MO, USA). Other Chemicals for HPLC separation were of the highest quality available.

2.2. Patients

The study was conducted on a 33-year-old male, whose clinical features, previously described [11], consist of podagra, asymptomatic uric acid stones, very mild psychomotor retardation and mild muscular hypertonia. His renal

function was normal (creatinine 0.74 mg/dl, creatinine clearance 83 ml/min.). He never experienced self-mutilation or choreoatetosis. The laboratory tests showed hyperuricemia, hyperuricaciduria, anemia and complete absence of erythrocyte HPRT activity [11]. Additional biochemical alterations in erythrocyte metabolism were also observed, confirming previous findings in LND [12] and were previously reported [11]. He also presented with G6PD deficiency and beta thalassemic trait. His mother developed hyperuricemia; one maternal and one paternal uncle suffer from gout by 67 and 61 years of age, respectively. The patient was under allopurinol treatment at 300 mg per day.

Control adults were 32 healthy volunteers 18–64 years of age (17 females and 15 males). All subjects gave their informed consent to the study. Blood samples as well as skin biopsies from the proband and controls were obtained as part of the treatment programme or provided by voluntary donors and thus ethical committee approval was not required.

2.3. Biochemical analysis: studies on blood

Red cells were isolated from venous blood anticoagulated with heparin by centrifugation (1500 × g, 5 min) and washed twice with isotonic NaCl solution (155 mM). The hematocrit of the packed cells was recorded for subsequent quantification of biochemical parameters. Perchloric extracts were obtained from 100 l packed erythrocytes for nucleotide analysis, and from 200 l plasma for metabolite analysis, as previously described [13]. Extracts were processed by HPLC or stored at –20 °C.

Cell-free lysates were obtained from washed erythrocytes by water dilution and freezing–thawing for the determination of enzyme activities of HPRT (with both hypoxanthine and guanine as substrates, the latter indicated as GPRT) and APRT, in erythrocyte lysates. Conversion of ¹⁴C-labelled precursors hypoxanthine (Hyp) and adenine (Ade) into their nucleotides was investigated after incubation of intact cells in an isotonic PPribP producing medium, in the presence of the appropriate precursor, for 1 h as previously described [12].

2.4. Biochemical analysis: studies on fibroblasts

Primary cultures of fibroblasts were obtained from skin biopsy of the patient and 4 controls with no neurological disease. Cultures were routinely grown at 37 °C in a humidified 5% CO₂ atmosphere in Dulbecco's modified minimal essential medium (DMEM) supplemented with L-glutamine, antibiotics and 10% foetal calf serum (FCS). At subconfluency both HPRT[–] and control cells were washed twice with PBS (without Mg⁺⁺ and Ca⁺⁺) and detached from the flasks by trypsinisation. Fibroblasts were washed in the medium, counted and resuspended at approximately 1–2 × 10⁶ cells/ml and used immediately for enzyme activity assay in lysates or for live fibroblast assay.

Cell-free lysates were obtained by freezing–thawing twice fibroblast suspensions in potassium phosphate buffer 10 mM pH 7.5; lysates were used for enzyme assays (HPRT, using either hypoxanthine or guanine as substrates, and APRT). Assay mixtures contained 55 mM Tris buffer pH 7.4, 5.5 mM MgCl_2 , 1 mM PPRibP, 0.3 mM α,β methylen-ADP, 0.6 mM either hypoxanthine, or guanine or adenine and suitable amount of cell lysate in a final volume of 200 μl . [^{14}C]-hypoxanthine and [^{14}C]-guanine (50 $\mu\text{Ci}/\mu\text{mole}$) were added in the patient enzyme assays. Perchloric extracts of the reaction mixtures were obtained after incubation for 20' at 37 °C, and used for HPLC analysis.

Intact fibroblasts ($0.2\text{--}2 \times 10^6$ cells) were incubated for 2 h at 37 °C in 2 ml Eppendorf tubes containing 200 μl of medium described above, with the appropriate radio-labelled substrate, [^{14}C]-hypoxanthine (Hyp), or [^{14}C]-guanine (Gua) or [^{14}C]-adenine (Ade) (0.1 mM) to evaluate base conversion into nucleotides. After incubation cells were spun down (10' at $250\times g$) and perchloric extracts of pelleted cells and supernatant fluid were processed by HPLC.

Protein content was measured in lysates and pelleted cells [20] for quantification of biochemical parameters.

Both non-radiochemical or radiochemical HPLC-linked methods previously described [12,13] were used with minor modifications to measure nucleotide content in erythrocyte and fibroblast extracts, nucleosides and bases in plasma extracts, as well as enzyme activities in cell lysates and radiolabeled base conversion by intact erythrocytes and fibroblasts. HPLC apparatus consisted of a Beckman System Gold Module 126, with a mod.168 Nouveau diode array detector (System 1), and a Beckman 420 controller, equipped with a mod. 167 dual channel scanning detector module (System 2) and an in-line mod. 171 radioisotope detector (Beckman San Ramon, CA, U.S.A.). Phenomenex Luna C18 columns (3 μm particle size, 75×4.6 mm) equipped with guard columns (Phenomenex Security guard 4 mm L \times 3 mm ID) were used. The absorbance was monitored at 260 and 280 nm. Retention time, coelution with added internal standards, absorption spectra and/or 260/280 nm absorbance ratios confirmed peak identities; concentration/area linear plots were developed for quantification. Radioactive compounds were separated by Sys 2; the UV traces at 260 and 280 nm and the radioactivity trace were followed.

2.5. Molecular (genetic) analysis

Peripheral blood samples were collected, after informed consent was obtained, in vacutainer tubes for mRNA analysis from proband and for genomic DNA analysis from proband and relatives (mother, father, sister, one maternal and one paternal uncles). Moreover, 50 healthy sardinian volunteers were analyzed to exclude a genetic polymorphism in sardinian population.

The sequence of the HPRT1 coding region was obtained by sequencing cDNA reverse-transcribed from RNA of the patient. Briefly, total RNA was extracted from 5 ml peripheral blood using a standard organic method (Trizol, Invitrogen) and alcoholic precipitation. Two ng of RNA were reverse-transcribed in a final volume of 20 ml containing 5 Units of AMV reverse transcriptase (Roche Molecular Biochem), 50 pmol of poliT primer, 0.2 mM of each dNTP in a buffer recommended by the manufacturer. Five μl of the transcript were then amplified using 50 pmol of the primers HPRT50: GCAGCCCTGGCGTCGTGAT and HPRT728: TGGCGATGTCAATAGGACTCCA, in a final volume of 50 μl containing $1 \times$ PCR buffer, 0.2 mM of each dNTP, 1.5 mM MgCl_2 and 1.25 units of Taq DNA Polymerase (AmpliTaQ Gold-Perkin Elmer). The PCR cycles consisted of an initial denaturation step of 95 °C for 5 min, followed by 40 amplification cycles (95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s). PCR product was purified and sequenced according to Sanger on an automated sequencer MegaBACE1000 (Amersham Pharmacia), following the instructions included in the DYEnamic ET Dye terminator cycle sequencing kit (Amersham-Pharmacia). Exons 1, 2 and 6 were analyzed by direct sequencing of the PCR products amplified from genomic DNA using the following primers: TGC TGC GCC TCC GCC TC and GAG CCC GCA CTG CGG ATC for exon 1; TGT ATC CTG TAA TGC TCT CAT TGA A and GAA CAG CTG CTG ATG TTT GAA for exon 2; GCA GTT ATA CAT GGG GGT TTT G and GCA ATC ACT TAA TCC CCC TTC for exon 6.

3. Results

The clinical history of the patient and the diagnostic biochemical data obtained in the first survey have been reported previously [11].

Plasma oxypurines pattern showed uric acid concentration within normal range (296 $\mu\text{mol/l}$; controls 261 ± 59) and grossly increased hypoxanthine and xanthine concentrations (73 and 185 $\mu\text{mol/l}$, respectively; controls 3.8 ± 2.9 and 1.7 ± 0.8 , respectively) as expected during allopurinol treatment, remarkable amounts of oxypurinol, allopurinol metabolite, were also found. Erythrocyte nucleotide pattern showed grossly increased NAD^+ and NADH concentrations (134 and 61 nmol/ml packed cells, respectively; controls 54 ± 23 and 29 ± 8 , respectively), which is a common finding in HPRT deficient patients [12]. NADP^+ concentration was higher and that of NADPH was lower than normal values (60 and 1 nmol/ml packed cells, respectively; controls 48 ± 11 and 15 ± 12 , respectively) due to G6PD deficiency demonstrated in this patient. Table 1 summarizes phosphoribosyltransferase activities measured in intact erythrocytes and fibroblasts and in lysates from both cell types. Phosphoribosyltransferase activity measured with both hypoxanthine and guanine was undetectable in erythrocyte lysates, while APRT activity

Table 1

Phosphoribosyltransferase activities in erythrocyte and fibroblast lysates and in intact cells of the propositus and controls

	Lysate			Intact cells		
	HPRT	GPRT	APRT	[¹⁴ C]Hyp	[¹⁴ C]Gua	[¹⁴ C]Ade
Patient erythrocytes	ud	ud	91	ud	nd	704
Control erythrocytes	112 ± 12	117 ± 30	21 ± 4	173 ± 57	nd	106 ± 27
Patient fibroblasts	ud	6.4	71.8	0.02	0.042	4.5
Control fibroblasts	86 ± 16	127 ± 54	171 ± 32	1.15 ± 0.70	0.94 ± 0.18	1.44 ± 0.53

Erythrocytes: enzyme activities in nmol/h/mg Hb; intact cell nucleotide production rate in nmol/h/ml packed cells. Fibroblasts: enzyme activities in nmol/h/mg protein; live cell nucleotide production rate in nmol/2 h/10⁶ cell.

ud: undetectable; nd: not determined.

was grossly increased. [¹⁴C]-hypoxanthine and -adenine incorporation by intact erythrocytes confirmed findings in lysates. Residual HPRT activity was evidenced in patient's fibroblast lysates when using guanine, but not hypoxanthine, as a substrate (5% of control values). Only live cell assay revealed conversion of hypoxanthine into nucleotides (1.7% of control values), guanine conversion again demonstrating a higher rate (4.5% of control values).

Sequencing analysis of cDNA from patient revealed a single base substitution C→T at cDNA base 463 in exon 6. This transversion (C463T) changes codon 155 from CCA to TCA, resulting in substitution of 155 proline to serine (P155S).

The exon six region of genomic HPRT1 DNA was sequenced in order to detect possible mutations in the family members, test the possibility of direct DNA analysis for the identification of carrier females and to exclude a genetic polymorphism in sardinian population. DNA sequencing in the mother confirmed heterozygosity for C463T mutation. The mutation was not present in the father, sister, paternal and maternal uncles nor in the 50 controls, all presenting the normal allele. This confirmed that gouty symptoms presented by maternal uncle was not on an enzymatic basis.

4. Discussion

Almost 300 different mutations [2,9,10] occurring throughout the coding region of HPRT gene have been described, but direct correlation with enzyme function is still difficult. Deletions and duplications within the gene have been demonstrated to cause complete enzyme deficiency and LND. Point mutations have been identified in patients with either partial or complete deficiency. Point mutations in the same position may result in different clinical syndromes, while mutations in different positions may yield in syndromes of equal severity [2]. Identification of mutation provides a poor tool to predict the phenotypic manifestation. Conserved amino acid substitutions alter HPRT protein conformation less severely than non-conserved substitutions. Usually, mutations leaving some residual enzyme activity are typically associated with less severe clinical manifestations. Mutations in the “evolutionary conserved” regions of the gene that constitute

functional important sites in the enzyme leads usually to complete loss of activity [14].

The described mutation 463 C to T in exon 6 results in a change of codon 155 from CCA to TCA and in 155 Pro to Ser substitution. This single base substitution reduces the HPRT enzyme activity to virtually undetectable levels in erythrocytes, but some 5% is preserved in fibroblasts. This mutation has not been reported previously in any HPRT deficient patient and, to our knowledge, this is the first Sardinian patient in whom a mutation of the HPRT gene causing activity deficiency has been investigated.

In Sardinian population, the prevalence rate for G6PD deficiency is 15% [15], and the expected incidence of coexistence of the two deficiencies is higher than observed, though no epidemiological survey has been conducted. Heterozygosis for G6PD deficiency is considered an advantage against malaria and it is conceivable that the combination of this defect with HPRT deficiency, causing purine intermediate raise may undo this benefit.

The substitution of proline, a heterocyclic amino acid, by serine, a hydrophilic amino acid, close to the binding-domain for hypoxanthine (spanning from amino acid 129 to 140), could determine a protein modification resulting in a virtually complete HPRT deficiency manifested in erythrocytes but rescued at a minimal extent in other cell types such as fibroblasts. Different stability of transcription products in different cell types might account for this finding; in particular shortened life-span would result in complete loss of partially active molecules in anucleated cells such as erythrocytes, while allowing some residual activity due to protein resynthesis in nucleated cells.

The link between enzyme deficiency and a severe CNS disorder such as LND is still unclear. Increasing evidence points to neurotransmitter changes [16]. Increased urine excretion of the serotonin metabolite 5-hydroxyindoleacetic acid [17], decreased neuronal dopamine activity and reduced density of dopamine containing neurons have been described. Positron emission tomography scanning has shown deficiency in the dopamine transporter [18,19]. Generally accepted and not mutually exclusive hypotheses to explain the disorder onset include: GTP depletion, NAD/ATP depletion [20], retarded cell division during brain development, altered levels of cGMP, increased concentrations of xanthine and hypoxanthine in the central nervous

system, and altered levels of other neurotransmitters. The involvement of NAD is manifested by accumulation in the erythrocytes, possibly due to increased synthesis [12]; these cells are unable to drive its utilization through polyADPriboseylation reactions, but upregulation of such pathway has been suggested by studies in nucleated cells [20].

Complete HPRT deficiency in the erythrocytes of a patient without the usual devastating neurological symptoms confirms the need for investigating different cell types regarding residual HPRT activity and severity of clinical phenotype. Such investigation may add further information to conventional blood analysis and yield different conclusions, as in the present case. This is important for both prognosis and understanding pathogenesis in HPRT deficiency. Our patient does not experience a full spectrum of LND and should be included among the variant forms with mild neurological symptoms [2,10].

Further studies such as a multicentre survey gathering the patients sharing this atypical manifestation with our patient [21,22] may contribute to understand the mechanism of neurological dysfunction in HPRT deficiency.

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